The EMBO Journal (2005) 24, 824–835 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
www.embojournal.org

THE
EMBO
JOURNAL

Staphylococcus aureus RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression

Eric Huntzinger[1,5], Sandrine Boisset[2,5], Cosmin Saveanu[3], Yvonne Benito[2], Thomas Geissmann[1], Abdelkader Namane[4], Gérard Lina[2], Jerome Etienne[2], Bernard Ehresmann[1], Chantal Ehresmann[1], Alain Jacquier[3], François Vandenesch[2,*] and Pascale Romby[1,*]

[1]UPR 9002 du CNRS, Institut de Biologie Moléculaire et Cellulaire, Strasbourg Cedex, France, [2]Faculté de Médecine Laennec, National Reference Center for Staphylococci, INSERM E0230, Lyon Cedex, France, [3]URA2171-CNRS-Génétique des Interactions Macromoléculaires, Paris Cedex, France and [4]Plateforme protéomique, Institut Pasteur, Paris Cedex, France

Staphylococcus aureus **RNAIII is one of the largest regulatory RNAs, which controls several virulence genes encoding exoproteins and cell-wall-associated proteins. One of the RNAIII effects is the repression of *spa* gene (coding for the surface protein A) expression. Here, we show that *spa* repression occurs not only at the transcriptional level but also by RNAIII-mediated inhibition of translation and degradation of the stable *spa* mRNA by the double-strand-specific endoribonuclease III (RNase III). The 3′ end domain of RNAIII, partially complementary to the 5′ part of *spa* mRNA, efficiently anneals to *spa* mRNA through an initial loop–loop interaction. Although this annealing is sufficient to inhibit *in vitro* the formation of the translation initiation complex, the coordinated action of RNase III is essential *in vivo* to degrade the mRNA and irreversibly arrest translation. Our results further suggest that RNase III is recruited for targeting the paired RNAs. These findings add further complexity to the expression of the *S. aureus* virulon.**

The EMBO Journal (2005) **24,** 824–835. doi:10.1038/sj.emboj.7600572; Published online 27 January 2005
Subject Categories: RNA; microbiology & pathogens
Keywords: mRNA degradation; post-transcriptional control; regulatory RNA; RNase III; *Staphylococcus aureus*

*Corresponding authors. P Romby, UPR 9002 du CNRS, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France. Tel.: +33 388 41 70 51; Fax: +33 388 60 22 18; E-mail: p.romby@ibmc.u-strasbg.fr or F Vandenesch, Faculté de Médecine Laennec, National Reference Center for Staphylococci, INSERM E0230, IFR62, 7 rue Guillaume Paradin, 69372 Lyon Cedex 08, France. Tel.: +33 478 77 86 57; Fax: +33 478 77 86 58; E-mail: denesch@univ-lyon1.fr
[5]These authors contributed equally to this work

Received: 16 September 2004; accepted: 10 January 2005; published online: 27 January 2005

Introduction

Virulence gene expression in most bacteria is tightly regulated, and many bacterial pathogens turn on virulence genes under appropriate conditions allowing the bacteria to survive within the host and to evade the host defense system. Recent studies performed on pathogenic bacteria point to the importance of RNAs in virulence control. Regulatory RNAs might exert their activity in *trans* through antisense properties or by sequestering proteins (for a review, see Johansson and Cossart, 2003; Storz *et al*, 2004). One of the most attractive examples is the *Staphylococcus aureus* 514-nucleotide regulatory RNAIII, which regulates the expression of several virulence factors. *S. aureus* virulence factors are precisely controlled in response to cell density (quorum sensing), energy availability, and various environmental signals (reviewed in Novick, 2003). Signal receptors are the primary regulatory mediators for the expression of the virulon in *S. aureus*. Among these receptors, the *agr* system, composed of two divergent transcription units, functions as a sensor of the population density. Its operon combines a density-sensing cassette (*agrD* and *B*) and a two-component sensory transduction system (*agrA* and *C*). The P2 operon expression is required for transcriptional activation of the P3 operon coding for RNAIII, the intracellular effector of the *agr* regulon (Novick, 2003). The expression of RNAIII is maximal in the late logarithmic and stationary phase cultures.

RNAIII has the unique property of acting as a messenger RNA encoding *hld* (delta-hemolysin), and having multiple regulatory functions: repression of the expression of surface proteins such as protein A during the exponential phase, and activation of the expression of extracellular toxins and enzymes during the postexponential phase (Janzon and Arvidson, 1990; Kornblum *et al*, 1990; Novick *et al*, 1993). The secondary structure of RNAIII is characterized by 14 stem–loop structures and by two long helices, which close off structural domains (Benito *et al*, 2000). Only two specific domains of RNAIII have been shown to control the expression of different targets. The 5′ end of RNAIII positively regulates the translation of *hla* (encoding alpha hemolysin) by competing directly with an intramolecular RNA secondary structure that sequesters the *hla* ribosomal binding site (Novick *et al*, 1993; Morfeldt *et al*, 1995). The 3′ domain of RNAIII is necessary and sufficient for repression of protein A synthesis (Novick *et al*, 1993; Benito *et al*, 1998, 2000). In this latter case, RNAIII is believed to function either directly or indirectly at the transcriptional level (Patel *et al*, 1992; Novick *et al*, 1993; Cheung *et al*, 1997). However, one cannot exclude an alternative mechanism in which RNAIII would alter the stability of *spa* mRNA (Novick *et al*, 1993; Benito *et al*, 2000). Indeed, sequence complementarity between RNAIII and *spa* mRNA has been suggested previously (Novick, 2003).

The present study analyses the regulatory mechanism by which RNAIII inhibits the expression of the surface protein A, one of the major virulence factors. Our results indicate that *spa* mRNA is repressed at the translational level by RNAIII and, in turn, is rapidly degraded by the double-strand-specific endoribonuclease III (RNase III). The mechanism of RNAIII action relies on an antisense activity, which is located in the 3′ domain and, is mediated by base pairings with *spa* mRNA.

Results

spa regulation occurs both at the transcriptional and post-transcriptional levels in vivo

Sequence complementarity between RNAIII (nucleotides 384–445) and *spa* mRNA (nucleotides 2–58) suggested that the 3′ domain of RNAIII can repress *spa* expression at the translational level, through annealing to *spa* mRNA (Novick, 2003). In order to test the *in vivo* relevance of such a mechanism, we fused the *spa* and *lacZ* genes in expression vectors. The *spa* promoter region (−454 to +1) alone or containing part of the coding sequence (−454 to +12) were cloned into the pTCV-*lac* shuttle vector (Poyart and Trieu-Cuot, 1997). We also used an *agr*-independent promoter (P*rpo*B) fused to the coding sequence of *spa* mRNA (+1 to +63), which contains the sequence complementarity to RNAIII. These constructs were designed in order to distinguish between transcriptional and post-transcriptional control. β-galactosidase activity was assayed in the *S. aureus* strains RN6390 (*agr*+) and WA400 (Δ*rnaIII*). Experiments were also carried out on the strain WA400, complemented with different sets of plasmids expressing either the wild-type RNAIII (LUG581) or RNAIII-Δ1 (LUG451) deleted of residues 430–437, which are complementary to the Shine and Dalgarno sequence of *spa* mRNA (see Figures 1 and 2A).

We observed that the minimal *spa* promoter element, which responds to the *agr* regulation, required the first 12 nucleotides of the *spa* gene in agreement with recent data showing that *agr* response involved nucleotides −137 to +7 (Gao and Stewart, 2004; Figure 1A). As expected, under the control of the *spa* promoter, the expression of the fusion protein strongly decreased in the wild-type strain as compared to the mutant strain WA400 (Figure 1A). These data suggest that RNAIII is required for the transcriptional control of *spa* expression.

When the *rpoB* promoter was fused with the *lacZ* gene, the β-galactosidase synthesis was identical in RN6390 (*agr*+) and WA400 (Δ*rnaIII*) showing that the *rpo*B promoter activity is *agr* independent (Figure 1A). The addition of the first 63 nucleotides of *spa* mRNA fused in frame with *lacZ* deleted of its ribosomal binding site (ΔRBS) reduced the β-galactosidase activity by two-fold in the wild-type strain as compared to WA400 (Figure 1B). The β-galactosidase levels decreased more drastically in WA400 carrying the multicopy plasmid pE194 producing high level of the wild-type RNAIII (LUG581) (Figure 1B). Conversely, the expression of RNAIII-Δ1 in WA400 (LUG451) had no effect on β-galactosidase reduction

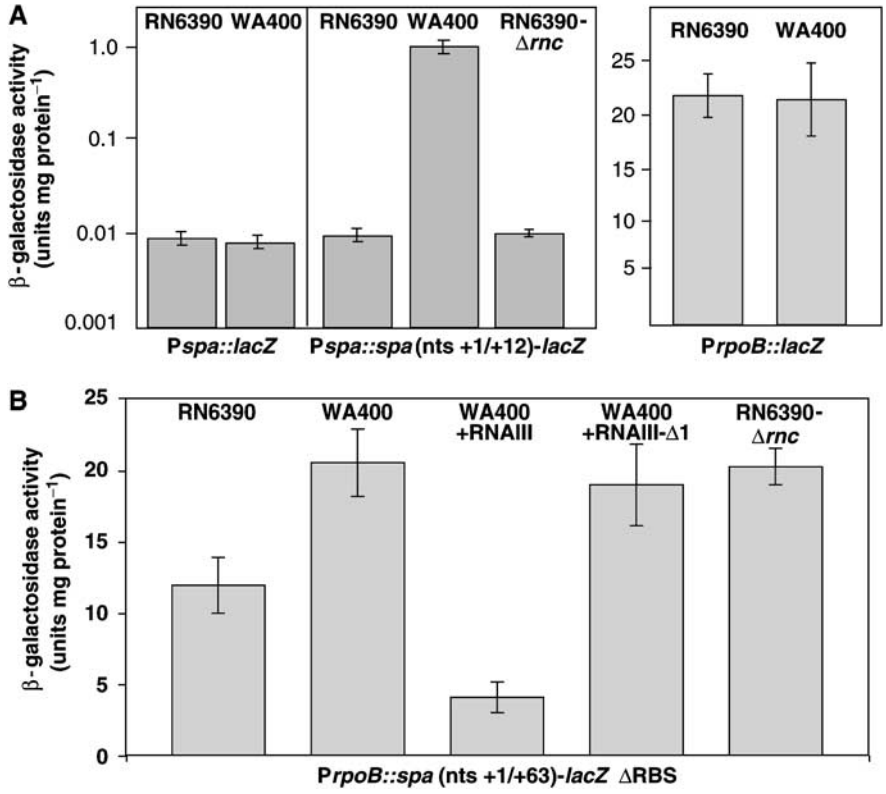

Figure 1 β-galactosidase activity detected from different gene fusions. (**A**) P*spa*::*lacZ* and P*spa* (+1/+12)::*lacZ* fusions in *S. aureus* RN6390 (*agr*+, WT), WA400 (Δ*rnaIII*) and RN6390-Δ*rnc* (LUG774, Δ*rnc*), and P*rpoB*::*lacZ* fusion in *S. aureus* RN6390 and WA400. (**B**) P*rpoB*::*spa* (+1/+63)-*lacZ* fusion in different *S. aureus* strains: RN6390, WA400, WA400 + RNAIII (LUG581, Δ*rnaIII*/pLUG298), WA400 + RNAIII-Δ1 (LUG451, Δ*rnaIII*/pLUG302) and LUG774. The β-galactosidase activity is expressed in arbitrary unit per milligram of protein. The results represented a mean of three independent experiments.

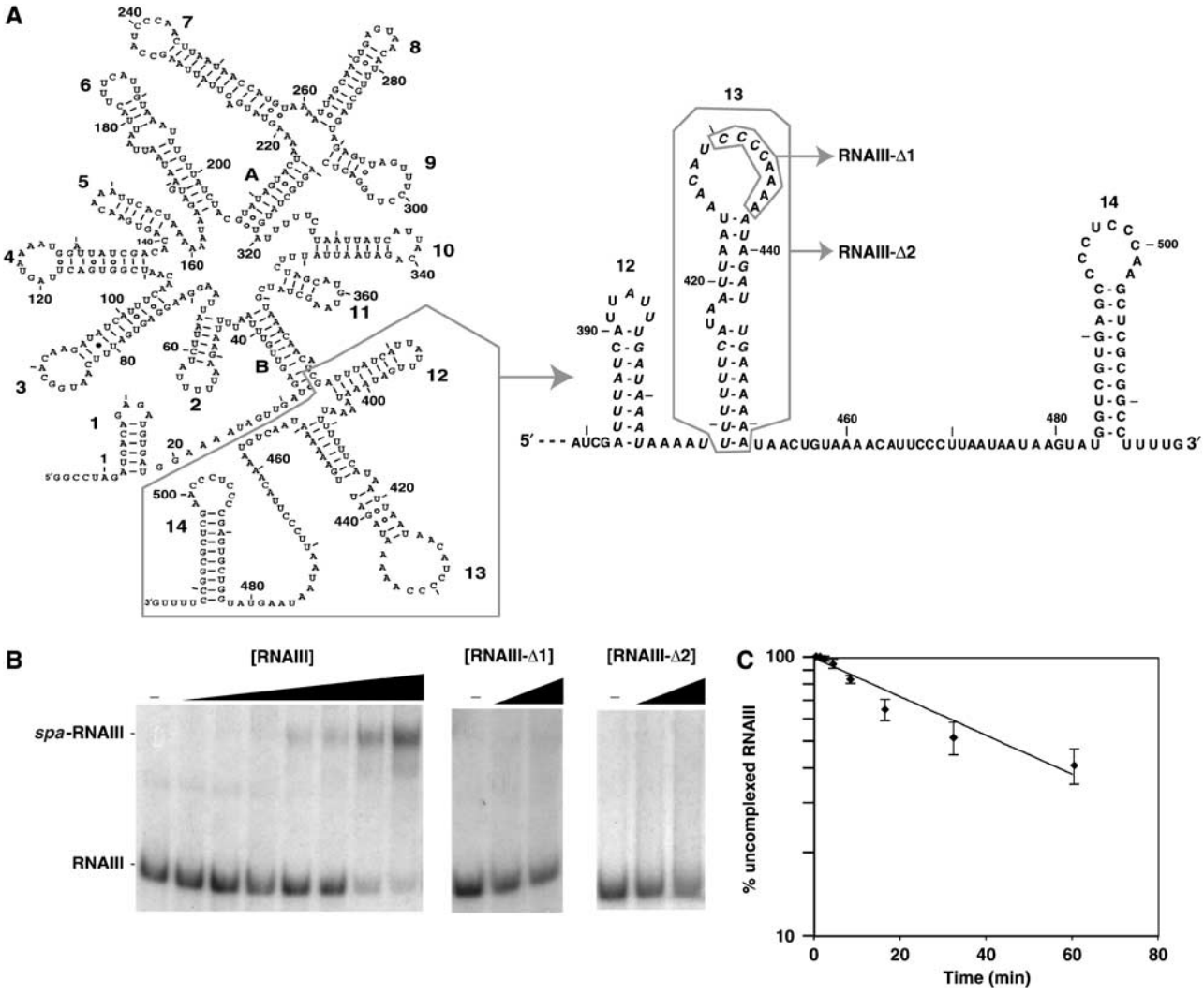

Figure 2 RNAIII binds efficiently to *spa* mRNA *in vitro*. (**A**) The secondary structure model of RNAIII from Benito *et al* (2000), and the 3′ domain are given. The two mutants, which carry deletion in loop 13 (RNAIII-Δ1), and the whole deletion of hairpin 13 (RNAIII-Δ2) are squared. Nucleotides complementary to *spa* mRNA are printed in italic. (**B**) Determination of the apparent dissociation constant for RNAIII/*spa* mRNA complex. The 5′-end-labeled *spa* mRNA was incubated alone (−) or with various concentrations of unlabeled wild-type RNAIII (0.1, 0.5, 1, 5, 10, 50 and 100 nM) and mutant RNAIII (50 and 100 nM). The fraction of labeled *spa* mRNA associated with RNAIII was calculated from the counts in the corresponding band relative to the total counts in the lane. The apparent K_d value was determined as the concentration of RNAIII allowing 50% of *spa* mRNA binding. (**C**) Binding rate constant for the RNAIII/*spa* mRNA complex as determined from three independent experiments. The 5′-end-labeled RNAIII (0.1 nM) was incubated with unlabeled *spa* mRNA (1 nM) at 37°C. Aliquots were withdrawn at 0, 1, 2, 4, 8, 16, 32 and 60 min. The percentage of free RNAIII was plotted as a function of time.

(Figure 1B). Taken together, these results indicate that *spa* expression is controlled at both transcriptional and post-transcriptional levels.

RNAIII interacts with spa mRNA in vitro

The *in vivo* data prompted us to examine whether RNAIII might efficiently bind to *spa* mRNA. Duplex formation between RNAIII and *spa* mRNA was investigated by gel shift analysis. *In vitro*-synthesized labeled *spa* mRNA (<1 nM) was incubated with increasing concentrations of unlabeled RNAIII in the presence of 5 mM $MgCl_2$ at 37°C for 15 min. The reactions were analyzed by gel electrophoresis using nondenaturing polyacrylamide gels (Figure 2B). RNAIII was found to bind *spa* mRNA with an apparent dissociation constant of 31 $(\pm 5.5) \times 10^{-9}$ M. The deletion of nucleotides C430 to A437 (RNAIII-Δ1) or of the whole hairpin 13 (RNAIII-Δ2) in RNAIII (Figure 2A) had a deleterious effect on complex formation (Figure 2B). Thus, RNAIII formed a moderately stable complex with *spa* mRNA and mutations, altering the complementarity with *spa* mRNA, impaired complex formation.

The rate of complex formation between RNAIII and *spa* mRNA was further analyzed by gel shift assays (Figure 2C). The initial rate of binding (k_{app}) was estimated by a time-course analysis using labeled RNAIII and an excess of the unlabeled *spa* mRNA and was determined to be 3 $(\pm 0.5) \times 10^5\,M^{-1}\,s^{-1}$. The rate of binding was proportional to the concentration of the RNAs, as expected for a second-order reaction (result not shown). This constant is probably underestimated since complex formation is reversible and some dissociation could occur within the gel. The value is, however, close to that obtained for OxyS-*fhlA* mRNA

(Argaman and Altuvia, 2000) and in the range of the association rate constants of 10^5–10^6 M^{-1} s^{-1}, determined in a variety of natural fully complementary antisense–target RNA systems using analogous methodology (for a review, see Wagner *et al*, 2002). This rapid binding between antisense and target RNA is thus compatible with RNAIII exerting its inhibitory effect on *spa* expression by binding to *spa* mRNA *in vivo*.

RNAIII sequesters the ribosome binding site of spa mRNA

The conformation of the first 200 nucleotides of *spa* mRNA either free or bound to RNAIII was probed using different enzymes and chemicals under conditions where less than one cleavage/modification per molecule takes place. Enzymes used were RNase T1 (specific for single-stranded guanines), RNase T2 (specific for unpaired nucleotides with a preference for adenines) and RNase V1 (specific for double-stranded regions). *Spa* mRNA was also subjected to chemical modifications by using DMS (A(N1), C(N3)) and CMCT (U(N3), G(N1)). Experiments are shown in Figure 3 and the reactivity levels are indicated on the secondary structure of the RNAs in Figure 4.

For RNAIII, the data were in good agreement with the secondary structure previously derived from chemical and enzymatic probing (Bénito *et al*, 2000; Figure 2A). The 3′ domain (containing hairpins 13 and 14) was shown to form an intrinsic structural domain, highly conserved among *Staphylococcus* species (Bénito *et al*, 2000). The existence of hairpin 13 (the main *spa* mRNA binding site) was supported by previous data: nucleotides within loop 13 were reactive at their Watson–Crick position despite their weak accessibility towards RNase T2, while the helix was cleaved by RNase V1 (Bénito *et al*, 2000; Figure 4B). For *spa* mRNA, the probing data were well correlated with the existence of hairpins I and II (Figure 4A). An alternative structure in *spa* mRNA involving nucleotides A29 to A47 is suggested due to the presence of several RNase V1 and RNase T2 cleavages (inset in Figure 4A).

Binding of RNAIII induced reactivity changes in a restricted region of *spa* mRNA, which covers the ribosome binding site. Protections against RNase T1 and T2 hydrolysis were mainly observed in loop I (Figure 4A). In addition, reactivity of adenines 29–33, and 35 towards DMS, and of uridines 42, 43, 46–47, 51–52 towards CMCT was significantly decreased upon RNAIII binding. Several RNase V1 cuts were also reduced at positions 18–20 and 26–27, whereas a new cleavage appeared at position 29. The reactivity pattern was unchanged in the other regions of *spa* mRNA.

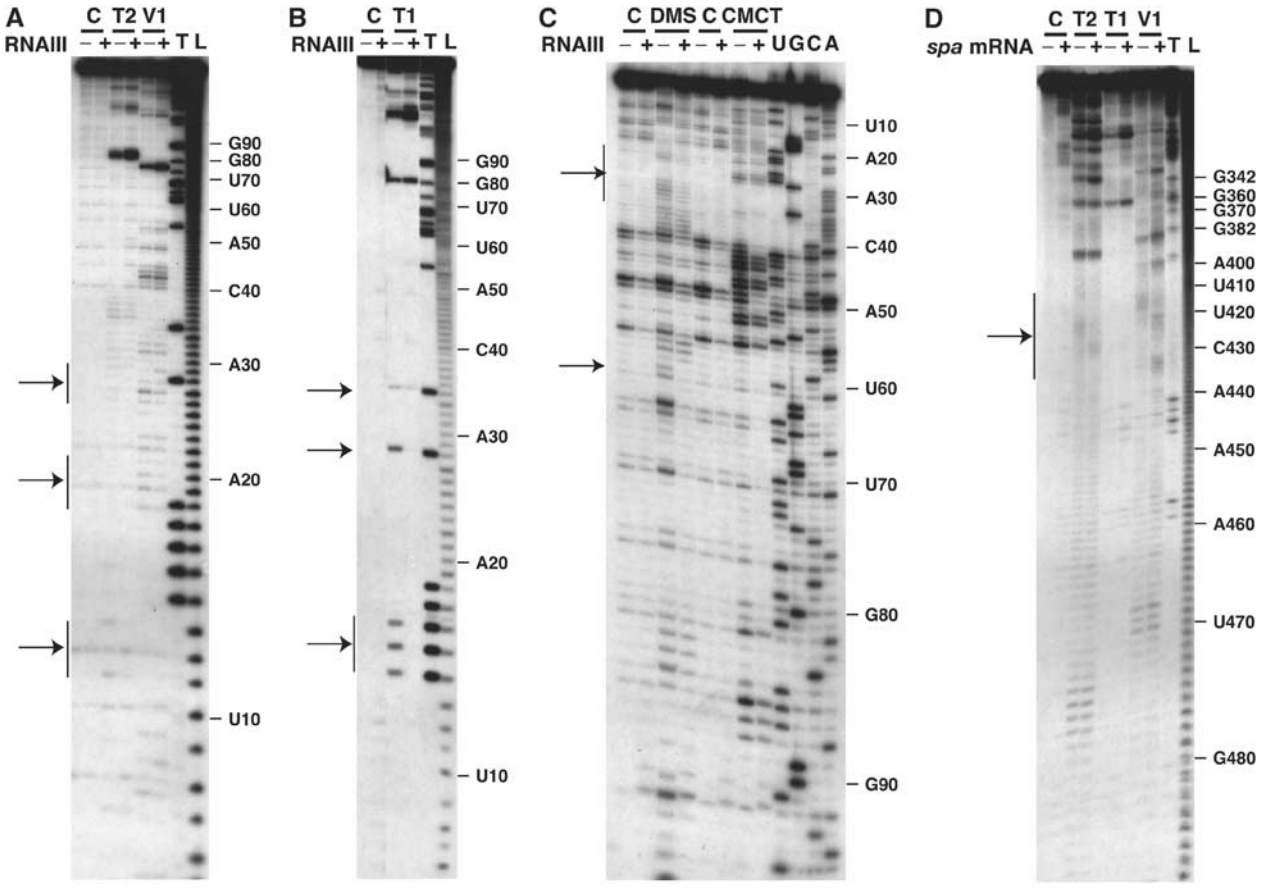

Figure 3 RNAIII binds to the SD region of *spa* mRNA. (**A, B**) Enzymatic hydrolysis of 5′-end-labeled *spa* mRNA, free (−RNAIII) or in the presence of an excess of RNAIII (+RNAIII). (**C**) Chemical probing of unlabeled *spa* mRNA, free (−) or in complex with RNAIII (+). Lanes U, G, C and A: dideoxy-sequencing reactions performed on *spa* mRNA. (**D**) Enzymatic hydrolysis of 3′-end-labeled RNAIII free (−*spa* mRNA) or in the presence of an excess of *spa* mRNA (+*spa* mRNA). T2, T1 and V1: RNase T2, RNase T1 and RNase V1, respectively. Lanes T and L: RNase T1 under denaturing conditions and alkaline ladders, respectively. Lanes C: incubation controls in the absence of the probe. Arrows denote the main reactivity changes induced by complex formation.

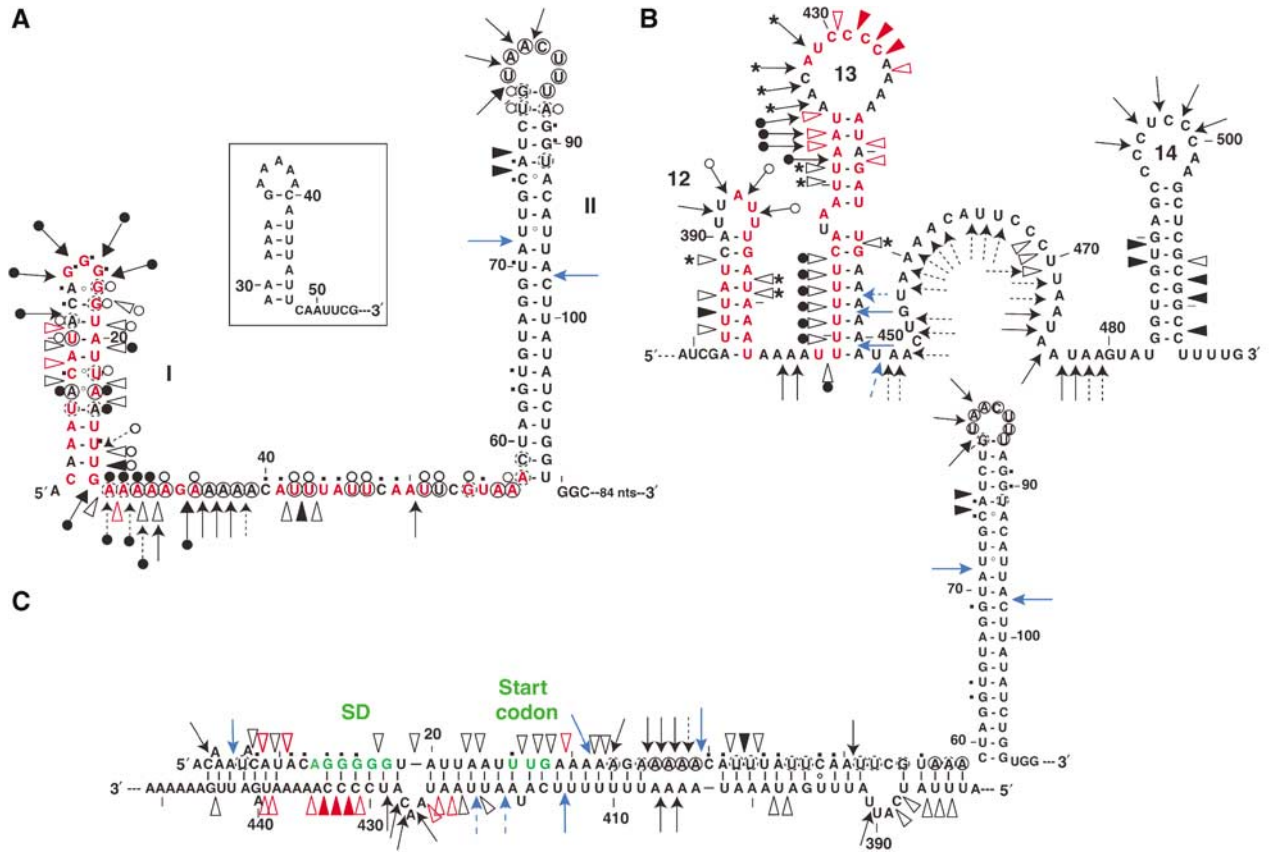

Figure 4 Secondary structure models of free and complexed RNA. A summary of the probing results is represented on the secondary structure of *spa* mRNA (**A**) and RNAIII (**B**). Enzymatic cleavages are given as follows: RNase T1 (⟶), RNase T2 (⟶) and RNase V1 (▷) moderate and (►) strong cleavage. Chemical modifications of cytosines at N3, and adenines at N1 towards DMS, and of uridines at N3 and guanines at N1 towards CMCT: full and dashed circled nucleotides are for strong and moderate reactivity, respectively. Small dots are for not determined due to unspecific cleavages or pauses of RT in the incubation control. Reactivity changes induced by complex formation are indicated as follows: black or empty circles denote strong and moderate protection, respectively; enhancements are represented by asterisks; new RNase V1 cleavages are shown by red arrows. Blue arrows show RNase III cleavages in the free RNA. Potential noncanonical base pairs are denoted by NoN. (**C**) Secondary structure model of the RNAIII/*spa* mRNA complex summarizing the enzymatic cleavages and chemical reactivities.

Binding of *spa* mRNA induced correlated reactivity changes in hairpin 13 of RNAIII, encompassing nucleotides 390–440 (Figure 4B). RNase T2 cleavages located at positions U392–U394 and U421–U424 were reduced, whereas increased cleavages were observed at A425–A428. New or increased RNase V1 cleavages also occurred at positions U420–U424, and C430–A434. The complex was also formed by annealing the two RNA molecules at high temperature followed by slow cooling to 37°C. The reactivity pattern was strictly identical to that of the complex formed at 37°C (results not shown).

These data show that RNAIII anneals to *spa* mRNA, and induces changes restricted to the complementary region that overlaps the ribosome binding site.

Binding of RNAIII to spa mRNA prevents the formation of the translational initiation complex

Since RNAIII binds to the Shine and Dalgarno sequence of *spa* mRNA, we tested the possibility that RNAIII binding is sufficient to prevent the formation of the ternary initiation complex formed by the *Escherichia coli* 30S subunit, the initiator $tRNA^{Met}$ and *spa* mRNA. The formation of the ternary complex blocked the elongation of a cDNA primer by reverse transcriptase, and produced a toeprint 16 nucleotides downstream of the initiation codon (Hartz *et al*, 1988; Figure 5A). Since RNAIII contained an effective ribosomal binding site that could interfere with ribosome binding at *spa* mRNA, we also used the two RNAIII mutants (RNAIII-Δ1, RNAIII-Δ2), which have lost the capacity to bind *spa* mRNA but are still recognized by the ribosome. The data revealed that the addition of increasing concentrations of RNAIII significantly decreased the yield of the toeprint (Figure 5A, lanes 6–8), 50% of inhibition being observed at the highest RNAIII concentration (Figure 5B). As the ternary 30S-mRNA–tRNA complex forms irreversibly, the inhibition was only observed when the initiator tRNA was added after RNAIII. The addition of RNAIII-Δ1 or RNAIII-Δ2 slightly altered ribosome binding since less than 20% of inhibition was observed at the highest concentration of the RNAIII mutants (Figure 5B). Thus, the specific RNAIII inhibition of ribosome binding to *spa* mRNA did not mainly result from a sequestration of the 30S subunit by RNAIII but rather from a direct interaction between *spa* mRNA and RNAIII. The binding of RNAIII to *spa* mRNA was sufficiently rapid to inhibit the formation of the translational initiation complex, at a step preceding the formation of the ternary complex.

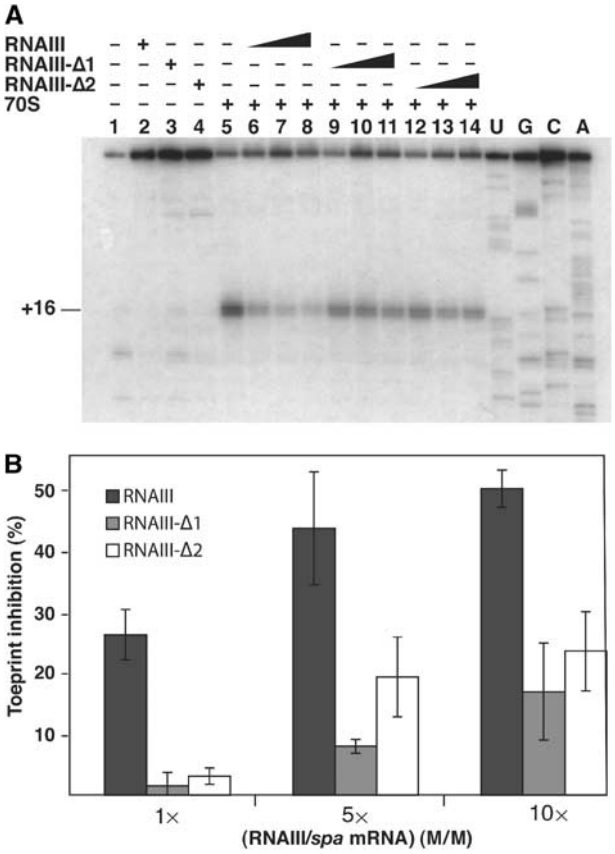

Figure 5 RNAIII prevents the formation of the ternary complex 30S-*spa* mRNA–tRNA. (**A**) Formation of the ternary complex between *spa* mRNA (15 nM), 30S ribosomal subunits (500 nM) and initiator tRNA (1 μM) was monitored in the absence (lane 5) or in the presence of wild-type RNAIII (lanes 6–8), RNAIII-Δ1 (lanes 9–11) and RNAIII-Δ2 (lanes 12–14). Concentrations of RNAIII species were 15, 75 and 150 nM. The toeprint at position +16 is indicated. Lanes U, G, C and A: dideoxy-sequencing reactions performed on *spa* mRNA. (**B**) Quantification of the data determined for three independent experiments. Relative toeprinting (toeprint band over full-length RNA + toeprint) was calculated by scanning of the gel with the Bio-imager Analyzer BAS 2000 (Fuji). The inhibition, given in %, represents the ratio between the relative toeprint in the presence of either the wild-type RNAIII or the mutant RNAs divided by the relative toeprint in the absence of RNAIII.

S. aureus RNase III is essential for efficient regulation of protein A expression in vivo

Many antisense-target systems are processed by the double-strand-specific RNase III (for a review, see Wagner *et al*, 2002). To test whether RNase III is involved in the regulation of *spa* expression, we constructed a strain derived from RN6390 in which the RNase III gene (*rnc*) was inactivated (LUG774). Codons 56–200 of the *rnc* gene, encompassing the RNA binding domain and the catalytic site, were deleted and replaced by the *cat* gene. Allelic replacement of the *rnc* gene in the strain RN6390 (forming LUG774) was analyzed by PCR using different sets of oligodeoxyribonucleotides, allowing detection of the expected sizes of the generated PCR fragments (data not shown). In contrast to *Bacillus subtilis* (Herskovitz and Bechhofer, 2000), the *rnc* gene is nonessential in *S. aureus*.

The translational fusion of the *spa* gene (1 to +63) with the *lacZ* gene expressed from the *agr*-independent P*rpoB* promoter was then transformed into LUG774 in order to analyze the post-transcriptional regulation of *spa* in the absence of RNase III. The β-galactosidase levels were identical to those measured in the mutant strain WA400 (Δ*rnaIII*) (Figure 1B), showing that the absence of either RNAIII or RNase III abolished *spa* repression. In contrast, low β-galactosidase levels were detectable in LUG774 carrying the transcriptional P*spa*::*lacZ* fusion (Figure 1A). These data suggest that the regulation of *spa* expression at the post-transcriptional level depends on both the presence of RNAIII and the double-strand-specific RNase.

S. aureus RNase III and RNAIII promote rapid degradation of repressed spa mRNA

We then tested the steady-state level of *spa* mRNA in the different *S. aureus* strains. Disappearance of *spa* mRNA was observed in RN6390 (*agr*+, Figure 6A, lane 1) as well as in

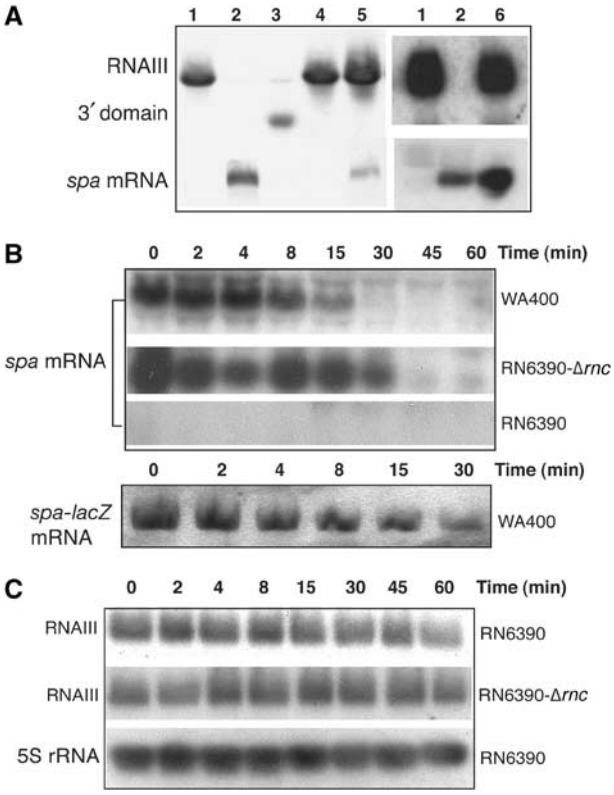

Figure 6 Analysis of *spa* mRNA and RNAIII levels in different strains. (**A**) Northern blot analysis on *spa* mRNA. RNAs from postexponential phase cultures were hybridized with probes corresponding to RNAIII and *spa* mRNA. Lane 1, RN6390 (WT, *agr*+); lane 2, WA400 (Δ*rnaIII*); lane 3, WA400 + 3′ domain (LUG484: Δ*rnaIII*/pLUG324); lane 4, WA400 + RNAIII (LUG581: Δ*rnaIII*/pLUG298); lane 5, WA400 + RNAIII-Δ1 (LUG451: Δ*rnaIII*/pLUG302); lane 6, RN6390-Δ*rnc* (LUG774: Δ*rnc*). (**B**) Measurements of the half-life of *spa* mRNA in the presence of rifampicin (300 μg/ml) in the strains WA400, RN6390-Δ*rnc* and RN6390. For comparison, the half-life of *spa-lacZ* mRNA (*agr*-independent P*rpoB* promoter) in WA400 is given. (**C**) Measurements of the half-life of RNAIII in the presence of rifampicin in the strains RN6390 (WT) and RN6390-Δ*rnc*. The half-life of 5S rRNA was measured on all the membranes in parallel, as an internal control. The quantity of *spa* mRNA or of RNAIII was normalized using the quantity of 5S rRNA at each time. The half-life was given as the time where 50% of the RNA was degraded. The experiments were reproduced four times.

WA400/pE194 expressing the wild-type RNAIII (LUG581, Figure 6A, lane 4). The level of *spa-lacZ* fusion mRNA (expressed under *agr*-independent *rpo*B promoter) was also strongly decreased in WA400/pE194 expressing the wild-type RNAIII (results not shown). Conversely, the level of *spa* mRNA was significantly increased in WA400 (Δ*rnaIII*) (Figure 6A, lane 2), and also in WA400/pE194 expressing RNAIII-Δ1, which has lost the capacity to bind *spa* mRNA (Figure 6A, lane 5). We also detected *spa* mRNA in the Δ*rnc* strain (LUG774) (Figure 6A, lane 6).

The half-life of *spa* mRNA was then analyzed in different strains (Figure 6B). In rifampicin-treated cells expressing RNAIII (RN6390, LUG581), *spa* mRNA was not detectable. Conversely, in rifampicin-treated cells, which do not express RNAIII (WA400), *spa* mRNA accumulated and was found to be unusually stable with a half-life of 15 (±2) min (Figure 6B). A long half-life was also measured for *spa-lacZ* mRNA (expressed under the *agr*-independent *rpo*B promoter), indicating that the 5′ leader conferred a high stability to *spa* mRNA (Figure 6B). Interestingly, degradation of *spa* mRNA was even reduced in Δ*rnc* strain (LUG774) since the half-life of *spa* mRNA increased to 32 (±2) min (Figure 6B). We also measured the half-life of RNAIII in the presence of rifampicin in RN6390 and LUG774. In both cases, RNAIII appeared to be highly stable (>45 min), as described previously (Janzon and Arvidson, 1990; Figure 6C), and no stable RNase III-specific processing intermediates were detectable in the wild-type strain (results not shown).

Since the *spa* mRNA half-life is long, we suggest that decay of *spa* mRNA significantly contributes to the overall repression mechanism, and that rapid degradation of *spa* mRNA is dependent on both RNAIII pairing and RNase III.

RNase III recognizes RNAIII, spa mRNA and the RNAIII–spa mRNA complex

Since RNase III is required for the post-transcriptional regulation of *spa* expression *in vivo*, we tested the capacity of the enzyme to cleave the duplex *in vitro*. The His-tagged RNase III from *S. aureus* was overexpressed in *E. coli* and purified. Mass spectrometry analysis and N-terminal sequencing did not reveal any contamination by *E. coli* RNase III. The extent of duplex formation was analyzed by comparing the RNase III-dependent cleavages under conditions of heat annealing and native RNAIII–*spa* mRNA complex formation. RNase III hydrolysis was conducted in parallel on the free RNA species. The cleavage positions were mapped using either end-labeled RNAIII or *spa* mRNA (Figure 7A and B), and are indicated on the secondary structure model (Figure 4C). The activity of *S. aureus* RNase III was dependent on Mg^{2+} ions since the presence of Ca^{2+} instead of Mg^{2+} in the reaction buffer inhibited the hydrolysis, as shown for the *E. coli* enzyme (for a review, see Nicholson, 1996).

Secondary structure analysis of *spa* mRNA supported a long and stable hairpin structure II, characterized by a 23 base pair (bp)-long helix (Figure 4A), which appeared to be a target site for *S. aureus* RNase III. Two main cleavages were detected at positions A71 and A97 (Figure 7A), even at low enzyme concentration. When labeled *spa* mRNA was incubated at 37°C in the presence of RNAIII to form the 'native' inhibitory complex, additional cleavages were detected at positions A5, A31 and A39 (Figure 7A).

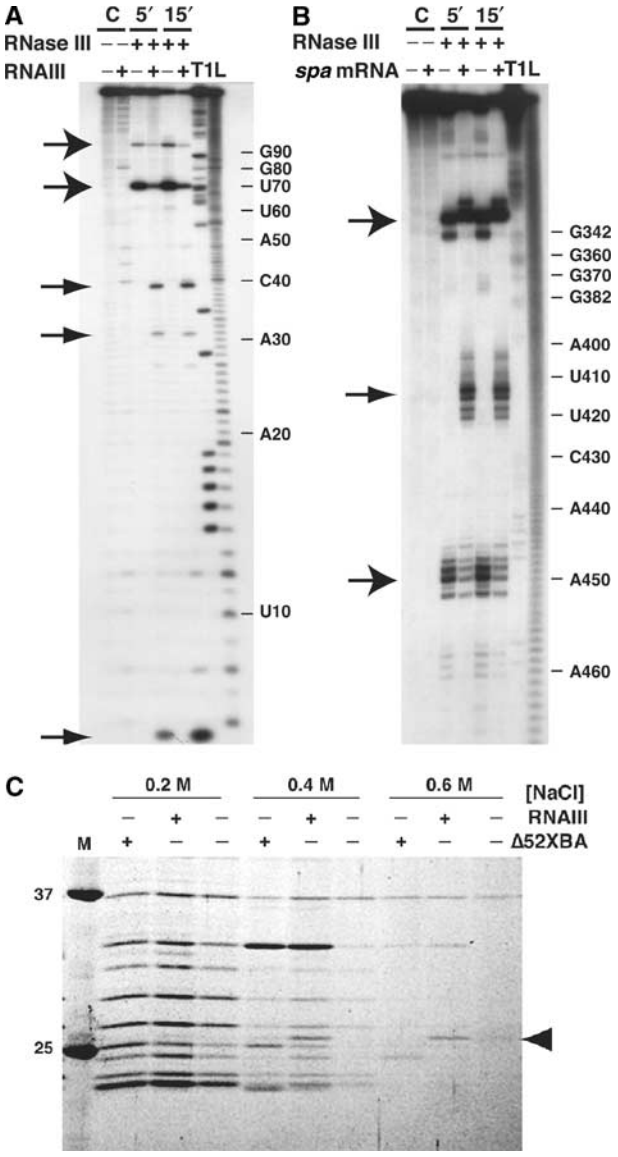

Figure 7 *S. aureus* RNase III recognizes the free *spa* mRNA and RNAIII, and RNAIII–*spa* mRNA complex. (**A**) RNase III hydrolysis of 5′-end-labeled *spa* mRNA, free (−) or in the presence of an excess of RNAIII (+). (**B**) RNase III hydrolysis of 5′-end-labeled RNAIII, free (−) or in the presence of an excess of *spa* mRNA (+). Lanes C: incubation control in the absence of RNase III. Lanes T1, L: RNase T1 and alkaline ladders, respectively. Arrows pointed the RNase cleavages occurring either in the free RNA (➔) or as the result of *spa*–RNAIII complex formation (➔). (**C**) *S. aureus* RNase III binds specifically to RNAIII. Crude extract was loaded onto the free streptavidin beads (−) and on the beads bound to either the 3′ end biotinylated RNAIII (+RNAIII), or a group II intron fragment RNA (Δ52XBA). Elution was carried out with increasing concentrations of NaCl. M is for molecular weight markers (in kDa).

Using end-labeled RNAIII, one major cleavage was reproducibly detected in the central domain of the free RNA, at position U321 and to a lesser extent in the 3′ domain, mainly at positions 448, A450 and U452 (Figure 7B). Binding of unlabeled *spa* mRNA induced new cleavages at positions U413 and to a lesser extent at C415, U418 and U420 within the region of complementarity with *spa* mRNA (Figure 4C). Concomitantly, cleavage at U321 persisted, while cleavages in hairpin 13 (A448, A450 and U452) were decreased

(Figure 7B). The same RNase III-induced cleavages were detected when the RNA–RNA complex was obtained by heat annealing, indicating that the native complex corresponds to the extended duplex.

In an attempt to identify *S. aureus* proteins that might specifically interact with RNAIII, we used *in vitro*-transcribed RNAIII, biotinylated at its 3′ end and immobilized on streptavidin–agarose beads. Among the proteins that showed specific association with RNAIII, a major band was identified by mass spectrometry as the RNase III. This protein was eluted from the RNAIII beads at high salt concentration only (0.6 M, Figure 7C) as compared with another RNA used as a control. To further confirm the identified interaction, we performed filter binding assays that showed an apparent dissociation constant of 0.5 μM for the RNAIII–RNase III complex, in the presence of Ca^{2+} to prevent hydrolysis (data not shown).

These data suggested that the *spa* mRNA–RNAIII complex formed an extended duplex recognized and cleaved by *S. aureus* RNase III. In addition, we found that *spa* mRNA alone, and more unexpectedly RNAIII, were specifically recognized by the double-strand-specific RNase III *in vitro*.

S. aureus Hfq may assist the RNAIII-dependent regulation

E. coli Hfq protein has been directly linked to the action of several small regulatory RNAs that use base pairings to regulate the expression of target mRNAs (e.g. Zhang *et al*, 2002). To test for an interaction between RNAIII and *S. aureus* Hfq protein *in vivo*, extracts from the wild-type cells (RN6390) were subjected to immunoprecipitation with either Hfq-specific serum or a nonspecific antibody. The experiments showed that RNAIII was only co-immunoprecipitated with Hfq-specific serum (Figure 8A). Neither the presence of RNAIII nor of Hfq was detected in the cell extracts using a nonspecific antibody. Using bandshift experiments, *S. aureus* Hfq was shown to bind RNAIII with a higher affinity than the regulatory region of the *spa* mRNA (Figure 8B and C). However, the addition of increasing concentrations of Hfq had no significant effect on the formation of the RNAIII–*spa* mRNA complex (Figure 8C).

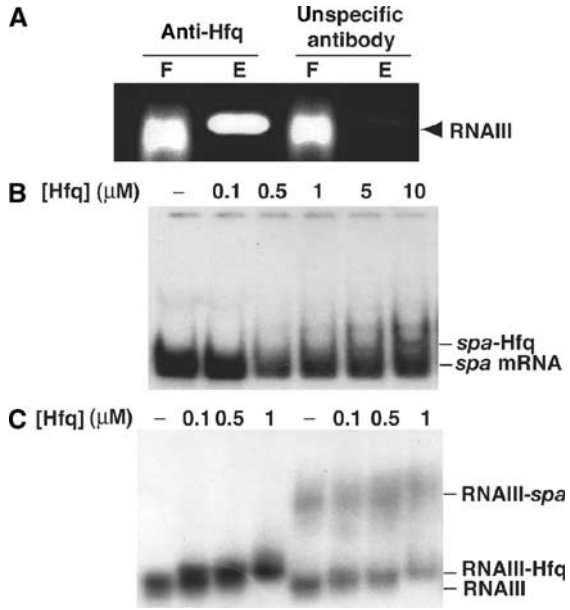

Figure 8 *S. aureus* RNAIII is a target of Hfq protein. (**A**) RNAIII co-immunoprecipitates with Hfq *in vivo*. RNAIII was detected by RT–PCR reaction in the flow through fraction (lane F) and in the precipitate fraction (lane E). The immunoprecipitation assays were also carried out with unspecific Ab, which do not bind Hfq. (**B**) Gel mobility shift assay with end-labeled *spa* mRNA in the presence of increasing concentrations of Hfq, and (**C**) with end-labeled RNAIII either free or in complex with *spa* mRNA in the presence of increasing concentrations of Hfq protein. Labeled RNAIII was first incubated with *spa* mRNA (10 nM) at 37°C for 20 min and various concentrations of Hfq (0.1, 5 and 1 μM) were added for a further 10 min of incubation. Note that the shift is not pronounced due to the large size of the RNAIII, but was reproducibly found in three independent experiments.

Discussion

S. aureus RNAIII is a regulatory RNA, which plays a key role in the quorum-sensing-dependent central regulatory circuit and coordinately regulates several virulence-associated genes (for a review, see Novick, 2003). The data presented here indicate that the RNAIII-dependent *spa* expression is not only repressed at the transcriptional level (Novick *et al*, 1993; Gao and Stewart, 2004) but also at the post-transcriptional level via an antisense RNA mechanism. Protein A is one of the major virulence factors, produced by almost all *S. aureus* clinical isolates, for which a tight regulation of expression might be critical. The membrane protein interferes with Ig-mediated opsonization (Patel *et al*, 1987) and is considered as a surface adhesin since it binds to the von Willebrand factor, a large glycoprotein mediating platelet adhesion at sites of endothelial damage (Hartleib *et al*, 2000; Gomez *et al*, 2004). Protein A has also the properties of a B-cell superantigen (Silverman and Goodyear, 2002) and acts as a natural B-cell toxin to induce the programmed cell death of susceptible VH-targeted B cells (Goodyear and Silverman, 2004). It is thus possible that during the infection process, the B-cell superantigen activity of protein A provides a benefit to the bacteria only when it is produced at low level. Conversely, a higher level of stimulation could trigger the innate immune system and may be deleterious for the bacteria. Such efficient down-regulation of protein A during the growth cycle and potentially during the course of infection suggests that the amount and timing need to be restricted to a narrow window for optimal efficiency. These observations might be correlated with the fact that the expression of the virulence factors follows a specific temporal program, suggesting that the adhesins and surface proteins are required earlier than the secreted postexponential enzymes, immunotoxins and cytotoxins in the course of the infection (for a review, see Novick, 2003). Notably, the expression of α-hemolysin, a postexponential exoprotein, is also regulated at both the transcriptional and translational levels (Novick *et al*, 1993; Morfeldt *et al*, 1995). This double control seems to be the rule rather than the exception for the expression of the virulence genes in *S. aureus*.

Transcription regulation of *spa* expression involves a complex interplay between positive (SarS, SarT, Rot) and negative (SarA) regulatory proteins (Novick, 2003). Based on the analysis of the regulatory elements of the *spa* promoter, a model was recently proposed for the regulation of *spa* transcription (Gao and Stewart, 2004). During early expo-

nential phase of growth, the *agr* system is turned off and as a consequence SarT and Rot become activated. These two regulatory proteins are required for the activation of SarS, which competes with SarA for binding to the *spa* promoter region to activate transcription (Gao and Stewart, 2004). In this scenario, one possible role of RNAIII would be to modulate the activity of transcriptional activators (Saïd-Salim *et al*, 2003). Alternatively, RNAIII could indirectly affect transcription through inhibition of mRNAs that encode transcriptional regulatory factors.

The 3′ domain of RNAIII causes direct repression of *spa* mRNA translation and induces its rapid degradation in a process that is dependent on the double-strand-specific RNase III. RNAIII acts as an antisense RNA and binds to *spa* mRNA with an association rate constant of $3 \times 10^5\,M^{-1}\,s^{-1}$. Rapid binding is a prerequisite for efficient control in many antisense regulatory systems, and is usually a consequence of a limited number of initial interactions that involve either a loop–loop or unpaired region–loop interactions (for a review, see Wagner *et al*, 2002). The deletion of the cytosine residues in loop 13 of RNAIII strongly decreased the association rate constant and impaired regulation *in vivo*. These data suggest that the initial binding involves a restricted number of nucleotides through a putative loop–loop interaction between loop 13 of RNAIII and loop I of *spa* mRNA (Figure 4). Probing experiments showed that the initial pairings are subsequently converted to extended interactions involving 35 bp interrupted by several bulged loops (Figure 4C). Since RNAIII sequesters the *spa* ribosome binding site, we propose that the primary effect of RNAIII is to prevent the formation of the translational initiation complex.

We also show that RNase III is essential for *spa* repression, and is involved in the degradation pathway of *spa* mRNA. In the wild-type strain, *spa* mRNA was not detectable, while in RNAIII-deleted strain, *spa* mRNA had a long half-life, which was even increased in the RNase III-deficient strain (Figure 6B). In *B. subtilis*, the initiation of mRNA decay is hindered by a 5′ proximal ribosome (Sharp and Bechhofer, 2003), and hairpin structures at or near the 5′ end interfere with initial degradation (Hambraeus *et al*, 2002; Condon, 2003). Thus, the presence of the hairpin I structure at the 5′ end of the *spa* mRNA, overlapping a strong ribosome binding site, may be responsible for its long half-life. Furthermore, we found that the long hairpin structure II located downstream of the ribosome loading site is an efficient target for RNase III *in vitro*, as well as the duplex formed by *spa* mRNA and RNAIII. Hence, the following mechanism can be proposed: at low cell density, *spa* mRNA is efficiently translated and protected from degradation; at high cell density, RNAIII synthesis is induced, allowing binding to *spa* mRNA and occlusion of the ribosome binding site. This inhibition of translation favors a rapid degradation of *spa* mRNA involving RNase III at the initial step. RNase III-induced cleavages in the formed duplex and in the long hairpin II of *spa* mRNA may render the mRNA accessible to other endo- or exoribonucleases for rapid degradation.

RNase III cleaves many different fully complementary antisense RNA/target mRNA duplexes, but these cleavages cause alterations in message decay rates in only few cases (Krinke and Wulff, 1990; Gerdes *et al*, 1997). Nevertheless, in most cases, translation inhibition essentially depends on the rate of the formation of RNA duplexes that remain stable for extended time periods. Thus, cleavage by RNase III of those mRNAs that form fully complementary antisense RNAs near or at the ribosome initiation site of the target mRNA do not significantly contribute to the efficiency of control (Case *et al*, 1990; Wagner *et al*, 1992). In the present case, *S. aureus* RNAIII also sequestered the Shine and Dalgarno sequence of the target *spa* mRNAs, and the association rate constant for complex formation suggested that inhibition might occur rapidly in a way similar to the fully complementary antisense RNAs. However, *in vitro* experiments indicated that the resulting complexes were moderately stable as compared to irreversible duplexes obtained from fully complementary antisense RNAs. Thus, we inferred that RNase III cleavage is required for rapid degradation and irreversible repression of *spa* translation.

Unexpectedly, we also showed that *S. aureus* RNase III specifically recognized the regulatory RNAIII. The functional significance of this interaction in the regulatory pathway of *spa* mRNA remains elusive. Given the length of RNAIII, one cannot exclude that RNase III might generate processing intermediates with different regulatory functions. However, RNAIII is highly stable in both the wild-type and RNase III-deficient strains, and no stable RNase III cleavage product intermediates were detected. Recently, Massé *et al* (2003) have shown that the RyhB RNA induced by iron starvation or other small noncoding RNAs induced by stress response, are rapidly degraded as a consequence of pairings with their target mRNAs, and thus act stoichiometrically. Since RNAIII is highly structured, RNase III may facilitate the coupled degradation of RNAIII and *spa* mRNA following complex formation.

Similar to *S. aureus* RNAIII, in *Streptococcus pyogenes* (Kreikemeyer *et al*, 2001; Mangold *et al*, 2004), and in *Clostridium perfringens* (Shimizu *et al*, 2002), small noncoding RNAs, induced by a two-component system, were shown to be involved in virulence gene expression. More recently, regulatory RNAs have been involved in the quorum-sensing cascade that regulates virulence in *Vibrio cholerae* and bioluminescence in *Vibrio harveyi* (Lenz *et al*, 2004). These regulatory RNAs, which target specific mRNAs, require the *trans*-acting protein Hfq in agreement with the fact that mutations in *V. cholerae hfq* gene produced strains defective in virulence (Ding *et al*, 2004). In *E. coli*, this Sm-like protein has been proposed to facilitate base pairings *in vitro* (Møller *et al*, 2002; Zhang *et al*, 2002; Geissmann and Touati, 2004), and to protect RNAs against RNase E-dependent degradation (Massé *et al*, 2003). We show here that RNAIII is a potential target of *S. aureus* Hfq *in vivo*, and that the protein binds more tightly to RNAIII than to the regulatory region of *spa* mRNA. The protein Hfq has, however, no detectable effect on RNAIII–*spa* mRNA complex formation *in vitro*, probably due to the fact that both RNAs interact with a fast association rate constant. Further experiments are underway to define more precisely the function of *S. aureus* Hfq in virulence, and the possible implication of the protein in conferring a high stability to RNAIII.

In summary, our data reveal an additional direct mRNA target for this unusually long regulatory RNAIII. How many genes are directly regulated by RNAIII and which mechanisms might be associated with them remains to be investigated.

Materials and methods

Strains, plasmids, construction of transcription–translation fusions

S. aureus RN6390, derived from 8325-4, is our standard agr^+ strain (Table I). *S. aureus* WA400 (Δ*rnaIII*) is a derivative of 8325-4, in which the P2 operon is functional but the P3 operon is deleted and replaced by the chloramphenicol transacetylase gene (*cat86*) (Janzon and Arvidson, 1990). The deletion/replacement Δ*rnc*::*cat* mutant of *S. aureus* RN6390 (LUG774) was obtained by using pMAD, a thermosensitive plasmid (Arnaud *et al*, 2004). Plasmid pTCV-*lac* is a low-copy-number promoter-less *lacZ* vector used for the study of promoter gene expression in Gram-positive bacteria (Poyart and Trieu-Cuot, 1997). A fragment containing the promoter of either the protein A gene (*spa*) encompassing nucleotides −454 to +1, −454 to +12, or the beta subunit of RNA polymerase gene (*rpoB)* encompassing nucleotides −480 to +1, were cloned in this vector. For transcriptional/translational fusions, plasmid pTCV-*lac* was modified by deletion of a region encompassing the Shine and Dalgarno sequence and the AUG sequence of *lacZ*. Detailed description is found in Supplementary data.

Northern blots and measure of mRNA half-life

Electrophoresis of total RNA was carried out on a 1% agarose gel containing 2.2 M formaldehyde and vacuum transfer to nylon membrane. Hybridizations with specific digoxigenin-labeled RNA probes and luminescent detection were carried out as described previously (Benito *et al*, 1998). Staphylococcal cultures at late exponential growth phase were treated with rifampicin (300 μg/ml) and incubated at 37°C. From 0 to 60 min, 1 ml of culture ($OD_{600}=1.5$) was taken at given times and the RNAs were extracted, and analyzed using specific probes to detect *spa* mRNA, *spa-lacZ* mRNA, RNAIII or 5S rRNA.

β-galactosidase measurements

S. aureus cells containing *lacZ* fusions were grown in BHI broth to exponential growth phase, washed and concentrated 10× (Sambrook *et al*, 1989). Cells (600 μl) were lysed using a FastPrep® Instrument (QBiogen). Protein concentrations were determined by the Bradford method. β-Galactosidase specific activity (Miller, 1972) was determined on 100 μl of lysate and expressed in arbitrary units per milligram of protein. All assays were carried out three times on duplicate cultures.

Table I Strains and plasmids

	Relevant characteristics	Reference or source
S. aureus strains		
8325-4	NCTC8325 cured of three prophages	Novick (1963)
RN4220	Restriction mutant of 8325-4	Kreiswirth *et al* (1983)
RN6390	Derivative of 8325-4, *agr* positive	Peng *et al* (1988)
WA400	8325-4: Δ*rnaIII* region::*cat86*	Janzon and Arvidson (1990)
LUG451	WA400/pLUG302	This work
LUG453	WA400/pLUG304	This work
LUG460	WA400/pLUG310	Benito *et al* (2000)
LUG484	WA400/pLUG324	This work
LUG581	WA400/pLUG298	This work
LUG754	RN6390/pLUG299	This work
LUG755	WA400/pLUG299	This work
LUG774	RN6390: Δ*rnc* region::*cat86*	This work
LUG779	WA400/pLUG516	This work
LUG780	WA400/pLUG517	This work
LUG785	RN6390/pLUG516	This work
LUG787	WA400/pLUG520	This work
LUG788	RN6390/pLUG520	This work
LUG790	RN6390/pLUG517	This work
LUG791	LUG451/pLUG520	This work
LUG792	LUG581/pLUG520	This work
LUG901	LUG774/pLUG516	This work
E. coli plasmids		
pQE30	6× His-tag plasmid	Qiagen
pLUG515	pQE30::*rnc* (nts 980–1670)	This work
pUT7-RNAIII	T7 promoter/RNAIII	Benito *et al* (2000)
pUT7-RNAIII-Δ1	T7 promoter/RNAIII-Δ1	This work
pUT7-RNAIII-Δ2	T7 promoter/RNAIII-Δ2	This work
pUT7-*spa*	T7 promoter/*spa*	This work
E. coli–staphylococcal shuttle plasmids		
pTCV-*lac*	Promoter-*lac* fusion shuttle vector: *spoVG-lacZ*, *ermB*, *aphA-3*	Poyart and Trieu-Cuot (1997)
pLUG220	pTCV-*lac*ΔRBS and start codon	This work
pMAD	Thermosensitive origin of replication, constitutively expressed *bga*B gene	Arnaud *et al* (2004)
pLUG519	pMAD derivative for deletion/replacement of *S. aureus rnc* gene	This work
Staphylococcal plasmids		
pE194	3.728 kb *S. aureus* plasmid, inducible MLS resistance (*erm*)	Horinouchi and Weisblum (1982)
pLUG274	pE194::*Eco*RV site in MCS	Benito *et al* (2000)
pLUG298	pLUG274::P3 operon (nts 1819–751)	This work
pLUG299	pTCVlac::P-*spa* (nts −454 to +1)	This work
pLUG302	pLUG274::*rnaIII*Δ (nts 430–437) (RNAIII-Δ1)	This work
pLUG304	pLUG274::*rnaIII*Δ (nts 408–451) (RNAIII-Δ2)	This work
pLUG324	pLUG274::P3 promoter (nts 1819–1569)::3′ end *rnaIII* region (nts 394–514 of RNAIII)	This work
pLUG516	pTCVlac::P-*spa* (nts −454 to +12)	This work
pLUG517	pTCVlac::P-*rpoB* (nts −480 to +1)	This work
pLUG520	pLUG220:P-*rpoB* (nts −480 to +1):*spa* (nt +2 to 63)	This work

Preparation of Hfq and immunoprecipitation

Recombinant Hfq, fused to maltose binding protein at its N-terminus, was overexpressed in *E. coli* and purified by affinity chromatography using an amylose resin. The fusion protein was eluted with 10 mM maltose, and the native Hfq protein was obtained after a specific proteolysis with the Xa factor. After purification on a mono-Q, Hfq was stored in a buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 250 mM NH_4Cl_2, 50% glycerol and 2 mM β-mercaptoethanol. Hfq-anti-serum was generated by immunizing two rabbits with purified Hfq protein.

For immunoprecipitation assays, *S. aureus* crude extract was first passed over a IgG sepharose 6FF column (Pharmacia) to avoid interference with the endogenous protein A. The clarified crude extract (100 µl) was incubated 2 h at 4°C with 5 µl rabbit serum containing *S. aureus* Hfq-specific antibody or with 5 µl anti-mouse antibody, respectively. The antibodies were then bound to 1 ml of protein A sepharose CL-4B equilibrated with 50 mM Tris–HCl, pH 8. After four washes with 2 ml of buffer containing 50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS, bound antibodies were eluted with 1 ml of 0.1 M glycine–HCl, pH 3, and neutralized with 100 µl of 1 M Tris–HCl, pH 8. RT–PCR reactions were carried out with two specific primers (RT-RNAIII5 and RT-RNAIII6, Supplementary Table S1).

Affinity chromatography using biotinylated RNAIII

In vitro-transcribed RNAIII (from plasmid pLUG322) and group II intron fragment Δ52XBA (as RNA control) were 3′ end biotinylated using biotin amidocaproyl hydrazide, and then bound to the streptavidin–agarose beads as described previously (Jestin *et al*, 1997). After incubation of the precleared *S. aureus* crude extract prepared from RN6390 strain, the proteins bound to the RNA beads were eluted by increasing NaCl concentration, fractionated on SDS–polyacrylamide gel and identified by MALDI TOF mass spectrometry (for details, Supplementary data).

RNase III preparation and RNA hydrolysis

The RNase III coding sequence was amplified from RN6390 gene sequence using primers rnase980/rnase1670 (Supplementary Table S1). The PCR product was digested with *Bam*HI and *Sma*I, ligated to the linearized pQE30 vector and transformed into *E. coli* M15. Recombinant RNase III, containing six histidines at its N-terminus, was purified by affinity chromatography using Ni^{2+} beads. The purified RNase III was stored in 25 mM Tris–HCl, pH 8.0, 150 mM KCl, 1 mM DTE, 0.1 mM EDTA and 50% glycerol at −20°C.

The 5′-end-labeled *spa* mRNA or RNAIII (3×10^{-8} M) were incubated with a five-fold excess of the unlabeled RNAIII or *spa* mRNA, respectively, in 10 µl TMN buffer containing 20 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 150 mM Na acetate and 1 mM DTT at 37°C for 15 min. Full RNA duplexes between RNAIII and *spa* mRNA were formed by incubation at 90°C for 2 min followed by slow cooling to 37°C in TMN buffer. Cleavages of end-labeled RNA, either free or in complex, were performed at 37°C from 30 s to 15 min with 8 pmol of RNase III in the presence of 1 µg of carrier tRNA. Reactions were stopped by phenol extraction followed by RNA precipitation.

Determination of constants of RNAIII/spa complex formation

Binding rate constant of RNAIII/*spa* complex was measured as described previously (Persson *et al*, 1988). For details see Supplementary data.

RNA structure probing and toeprinting

Enzymatic hydrolysis and chemical modifications on free or bound RNAs were performed as described previously (Benito *et al*, 2000). The formation of a simplified translational initiation complex with *spa* mRNA and the extension inhibition conditions were strictly identical to those described by Benito *et al* (2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank P Fechter, M Possedko, C Gaspin, H Moine, C Brunel for discussions. We are thankful to C Condon, G Wagner and C Isel for discussions and critical reading of the manuscript, to AC Helfer for her skilful technical help and to JC Cortay for technical advises. We are grateful to T Msadek and M Debarbouillé for providing us the pMAD plasmid and to P Trieu-Cuot and C Poyart for the pTCVlac vector. This work was supported by the Centre National de la Recherche Scientifique, the Ministère de la Recherche (ACI Microbiologie), the Ligue Régionale contre le Cancer and the Fondation pour la Recherche Médicale. SB is supported by a fellowship from the Fondation pour la Recherche Médicale.

References

Argaman L, Altuvia S (2000) *fhlA* repression by OxyS RNA: kissing complex formation at two sites results in a stable antisense–target RNA complex. *J Mol Biol* **300:** 1101–1112

Arnaud M, Chastanet A, Debarbouillé M (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70:** 6887–6891

Benito Y, Kolb FA, Romby P, Lina G, Etienne J, Vandenesch F (2000) Probing the structure RNAIII, the *Staphylococcus aureus agr* regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. *RNA* **6:** 668–679

Benito Y, Lina G, Greenland T, Etienne J, Vandenesch F (1998) *Trans*-complementation of a *Staphylococcus aureus agr* mutant by *Staphylococcus lugdunensis agr* RNAIII. *J Bacteriol* **180:** 5780–5783

Case CC, Simons EL, Simons RW (1990) The IS10 transposase mRNA is destabilized during antisense RNA control. *EMBO J* **9:** 1259–1266

Cheung AL, Eberhardt K, Heinrichs JH (1997) Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. *Infect Immun* **65:** 2243–2249

Condon C (2003) RNA processing and degradation in *Bacillus subtilis*. *Microbiol Mol Biol Rev* **67:** 157–174

Ding Y, Davis BM, Waldor MK (2004) Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. *Mol Microbiol* **53:** 345–354

Gao J, Stewart GC (2004) Regulatory elements of the *Staphylococcus aureus* protein A (*Spa*) promoter. *J Bacteriol* **186:** 3738–3748

Geissmann TA, Touati D (2004) Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J* **23:** 396–405

Gerdes K, Gultyaev AP, Franch T, Pedersen K, Mikkelsen ND (1997) Antisense RNA-regulated programmed cell death. *Annu Rev Genet* **31:** 1–31

Gomez M, Lee A, Reddy B, Muir A, Soong G, Pill A, Cheung A, Prince A (2004) *Staphylococcus aureus* portein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat Med* **10:** 842–848

Goodyear CS, Silverman GJ (2004) Staphylococcal toxin induced preferential and prolonged *in vivo* deletion of innate-like B lymphocytes. *Proc Natl Acad Sci USA* **31:** 11392–11397

Hambraeus G, Karhumaa K, Rutberg B (2002) A 5′ stem-loop and ribosome binding but not translation are important for the stability of *Bacillus subtilis aprE* leader mRNA. *Microbiology* **148:** 1795–1803

Hartleib J, Kohler N, Dickinson RB, Chatwal GS, Sixma JJ, Hartford OM, Foster TJ, Peters G, Kehrel BE, Herrmann M (2000) Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood* **96:** 2149–2156

Hartz D, McPheeters DS, Traut R, Gold L (1988) Extension inhibition analysis of translation initiation complexes. *Methods Enzymol* **164:** 419–425

Herskovitz MA, Bechhofer DH (2000) Endoribonuclease III is essential in *Bacillus subtilis*. *Mol Microbiol* **38:** 1027–1033

Horinouchi S, Weisblum B (1982) Nucleotide sequence and functional map of pC194, plasmid that specifies inducible chloramphenicol resistance. *J Bacteriol* **150:** 815–825

Janzon L, Arvidson S (1990) The role of the delta-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *EMBO J* **5:** 1391–1399

Jestin JL, Dème E, Jacquier A (1997) Identification of structural elements critical for inter-domain interactions in a group II self-splicing intron. *EMBO J* **16:** 2945–2954

Johansson J, Cossart P (2003) RNA-mediated control of virulence gene expression in bacterial pathogens. *Trends Microbiol* **11:** 280–285

Kornblum J, Kreiswirth B, Projan SJ, Ross H, Novick R (1990) *Agr*: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*. In: *Molecular Biology of the Staphylococci*, Novick RP (ed), pp 373–401. New York, NY: VCH Publishers

Kreikemeyer B, Boyle MDP, Buttaro BA, Heinemann M, Podbielski A (2001) Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (*fas*) with homologies to two-component-type regulators requires a small RNA molecule. *Mol Microbiol* **39:** 392–406

Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, Novick RP (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305:** 709–712

Krinke L, Wulff DL (1990) RNase III-dependent hydrolysis of lambda cII-O gene mRNA mediated by lambda OOP antisense RNA. *Genes Dev* **4:** 2223–2233

Lenz DH, Mok KC, Lilley BN, Kulkarni RV, Wingreen NS, Bassler BL (2004) The smalll RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* **118:** 69–82

Mangold M, Siller M, Roppenser B, Vlamickx BJM, Penfound TA, Klein R, Novak R, Novick RP, Charpentier E (2004) Synthesis of group A streptococcal virulence factors is controlled by a regulatory RNA molecule. *Mol Microbiol* **53:** 1515–1527

Massé E, Escorcia FE, Gottesman S (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia Coli*. *Genes Dev* **17:** 2374–2383

Miller JH (1972) Assays of lac operon enzymes: β-galactosidase permease, repressor, transacetylase, a complementation. In: *Experiments in Molecular Genetics*, Miller JH (ed), pp 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Møller T, Franch T, Højrup P, Keene DR, Bachinger HP, Brennan RG, Valentin-Hansen P (2002) Hfq: a bacterial Sm-like protein that mediates RNA–RNA interaction. *Mol Cell* **9:** 23–30

Morfeldt E, Taylor D, Von Gabain A, Arvidson S (1995) Activation of alpha-toxin translation in *Staphylococcus aureus* by the *trans*-encoded antisense RNA, RNAIII. *EMBO J* **14:** 4569–4577

Nicholson AW (1996) Structure, reactivity, and biology of double-stranded RNA. *Prog Nucleic Acid Res Mol Biol* **52:** 1–65

Novick RP (1963) Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *J Gen Microbiol* **33:** 121–136

Novick RP (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* **48:** 1429–1449

Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J* **12:** 3967–3975

Patel AH, Kornblum J, Kreiswirth B, Novick R, Foster TJ (1992) Regulation of the protein A-encoding gene in *Staphylococcus aureus*. *Gene* **114:** 25–34

Patel AH, Nowlan P, Weavers ED, Foster T (1987) Studies on the virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infect Immun* **55:** 3103–3110

Peng HL, Novick RP, Kreiswirth B, Kornblum J, Schlievert P (1988) Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J Bacteriol* **170:** 4365–4372

Persson C, Wagner EGH, Nordström K (1988) Control of replication of plasmid R1: kinetics of *in vitro* interaction between the antisense RNA, CopA, and its target, CopT. *EMBO J* **7:** 3279–3288

Poyart C, Trieu-Cuot P (1997) A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in Gram-positive bacteria. *FEMS Microbiol Lett* **156:** 193–198

Saïd-Salim B, Duman PM, McAleese FM, Macapagal D, Murphy E, McNamara PJ, Arvidson S, Foster TJ, Projan SJ, Kreiswirth BN (2003) Global regulation of *Staphylococcus aureus* genes by Rot. *J Bateriol* **185:** 610–619

Sambrook J, Fritsch E, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Press

Sharp JS, Bechhofer DH (2003) Effect of translational signals on mRNA decay in *Bacillus subtilis*. *J Bacteriol* **185:** 5372–5379

Shimizu T, Shima K, Yoshuno K, Yonezawa K, Shimizu T, Hayashi H (2002) Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. *Mol Microbiol* **43:** 257–265

Silverman GJ, Goodyear CS (2002) A model B-cell superantigen and the immunobiology of B lymphocytes. *Clin Immunol* **102:** 117–134

Storz G, Opdyke JA, Zhang A (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr Opin Microbiol* **7:** 140–144

Wagner EGH, Altuvia S, Romby P (2002) Antisense RNAs in bacteria and their genetic elements. *Adv Genet* **46:** 361–398

Wagner EGH, Blomberg P, Nordström K (1992) Replication control in plasmid R1: duplex formation between the antisense RNA, CopA, and its target, CopT, is not required for inhibition of RepA synthesis. *EMBO J* **11:** 1195–1203

Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G (2002) The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **9:** 11–22